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Copper-release from yeast Cu(I)-metallothionein by nitric oxide (NO)

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Abstract

The reaction of yeast Cu-MT with nitric oxide (NO) was examined. A release of copper from the Cu(I)-thiolate clusters of the protein by this remarkably important reagent was observed *in vitro*. The characteristic spectroscopic signals of the Cu(I)-thiolate chromophores levelled off in the presence of a two-fold molar excess of NO expressed per equivalent of thionein-copper as monitored by UV-electronic absorption, circular dichroism and luminescence emission. At the same time all of the copper became EPR detectable. The oxidized metal ions could easily be removed from the protein moiety by gelfiltration. The reversibility of the copper releasing process is of special interest. The specific fluorescence and dichroic properties of the previously demetallized protein could be recovered up to 85% under reductive conditions. Moreover, no difference in the electrophoretic behaviour was seen compared to the untreated Cu-MT. Thus, NO may act as a potent metabolic source for the transient copper release from Cu-MT. In the course of an oxidative burst this highly Fenton active copper is able to improve the efficacy of biological defence mechanisms.

Introduction

Our knowledge of the metabolic control of copper in biological systems is very limited. The essential role of this prominent transition metal demands a subtle regulation on a cellular level. 'Free copper ions' must be considered to be rather dangerous as there are many undesired reactions that would lead to the irreversible destruction of cellular components (Weser & Hartmann 1984; Felix & Weser 1988). In the course of an oxidative burst the controlled release of copper from Cu-metallothionein (Cu-MT) may be useful to improve the efficacy of the copper-driven reaction of oxygen free radicals. In this context Cu-MT has been regarded to be a promising candidate in the control of copper homeostasis (Weser & Hartmann 1984; Schechinger *et al.* 1986; Weser *et al*. 1986). Unlike the recent rigorously examined copper trafficking chaperones (Rae *et al.* 1999; Culotta *et al.* 1999; Valentine & Gralla 1997) a 'ferritin-like' action is attributed to Cu-MT. Furthermore, there are many other different possible functions of this Cu(I)-thiolate protein including the stoichiometric and catalytic reaction with oxygen free radicals and the control of intracellular redox potentials (Deters *et al.* 1994; Felix *et al.* 1993; Hartmann *et al.* 1984).

The question arises as to how the Cu(I)-thiolate bonding is formed and/or cleaved in this cysteine-rich protein. Several possibilities on the catabolism have been proposed (Weser *et al.* 1986). A controlled Cu(I) channelling off into the vacant copper-binding sites of apo-Cu-proteins was seen (Morpurgo *et al.* 1983; Hartmann *et al.* 1983). Proteolysis of Cu-MT proved successful only after prior destruction of the Cu-thiolate cluster (Weser *et al.* 1986). Numerous studies have been made on possible oxidation mechanisms induced by activated polymorphonuclear leukocytes (PMN's) (Hartmann *et al.* 1985, 1987; Schechinger *et al.* 1986), hydrogen peroxide and enzymes generating activated oxygen species (Hartmann *et al.* 1984; Richter & Weser 1988). S-alkylation of the Cu-thiolate clusters led to the release of copper from yeast Cu-

MT (Felix & Weser 1988). Treatment of this protein with hypothiocyanite ($OSCN^-$) which is formed in the presence of activated PMN's as a transiently abundant derivative of thiocyanate (SCN-), was found to break the Cu(I)-thiolate bonding with the consecutive generation of a reconstitutable apo-protein (Hartmann *et al.* 1996).

Nitric oxide (NO) is a reagent of remarkable importance which enjoyed considerable attention in recent years due to its diverse actions in biological systems (Snyder & Bredt 1992; Moncada & Higgs 1993; Traylor & Sharma 1992; Robbins & Grisham 1997). It is produced by a variety of cell types including endothelial cells, neutrophils, neurons and hepatocytes (Zhang & Snyder 1995; Barnes 1995). Interactions of NO with MT have recently been reported. NO induces zinc and /or cadmium release from the respective thiolate clusters of MT (Aravindakumar *et al.* 1999; Kroncke *et al.* 1994). The zinc finger-type yeast transcription activator LAC9, another zinc-thiolate protein, was inhibited after the NO-dependent removal of zinc (Kroncke *et al.* 1994). In addition, there is evidence that NO enhances cadmium toxicity by displacing the metal from MT in growing Chinese hamster ovary cells (Misra *et al.* 1996). At present, no data are available on the reactivity of NO with Cu-MT. Thus, it was of interest, whether or not copper can be released from yeast Cu-MT *in vitro* in the presence of this reagent. Unlike zinc and/or cadmium copper represents a redox metal which may react in a different way with NO.

The possible breakdown of the oligonuclear Cu(I)-thiolate centres was measured using UV-electronic absorption, circular dichroism (CD), luminescence emission and EPR-spectroscopy. Gelfiltration of NO-treated Cu-MT proved to be useful to monitor the possible release of copper from the protein moiety.

Materials and methods

Cu-MT from yeast (*Saccharomyces cerevisiae*) was isolated from the copper resistant strain X 2180-1Aa (obtained from J. Welch, Berkely, CA) after growing in a complete medium in the presence of 1 mM $CuSO_4$. Purification of the protein was performed by repeated gelfiltration of the homogenate supernatant on Sephadex G-50, equilibrated in 10 mM Tris/HCl, pH 7.4, 0.1% 2-mercaptoethanol (Weser & Hartmann 1991). NO gas was from Merck-Schuchardt, München. The concentration of NO in the reaction cuvette was estimated employing the spectrophotometrically detectable myoglobin/NO adduct formation (Kelm & Schrader 1988). Controlled bubbling of NO gas into a solution of myoglobin of known concentration and volume was used for calibration of the reagent. Electronic absorption was recorded on a Beckman 740 spectrophotometer. Luminescence emission was measured on a Perkin-Elmer LS 50 luminescence spectrometer. Excitation was at 300 nm. CD was run on a JASCO J 720 spectropolarimeter. Due to the lower intensity of the Cotton effects between 400 and 300 nm the more pronounced bands between 320 and 260 were chosen. CD spectra at lower wavelengths were not recorded as slight reagent-dependent perturbations were seen below 260 nm. X-band EPR spectra were run on a Bruker ESP 300 E spectrometer at 100 k. Gelfiltration of the NO treated Cu-MT was performed on H_2O equilibrated Sephadex G-25. Copper was quantified on a Perkin-Elmer 3030 atomic absorption spectrometer.

Results and discussion

The reactivity of NO with Cu-MT was examined. It was of interest whether or not this reagent may remove copper out of the oligonuclear Cu(I)-thiolate clusters of the protein. Yeast Cu-MT was chosen as it is well known to be homogeneous with respect to copper. In addition, this MT is substantially more stable against oxidation compared with the mixed Cu,Zn-MT from vertebrate origin. Thus, yeast Cu-MT was most appropriate to study in a model-type reaction the reactivity of NO on the Cu(I) thiolate binding centres which are similar in either α-and β-domain of copper-substituted vertebrate MT-species (Hartmann *et al.* 1992). The characteristic spectroscopic properties including UV-electronic absorption, CD and luminescence emission were used as sensitive tools for monitoring the intactness of the Cu(I)-thiolate clusters.

Equimolar NO expressed per equivalent of thionein-copper diminished the intensity of the luminescence emission by 75–80% within seconds. In the presence of a two-fold molar excess the signal levelled off completely (Figure 1). A similar behaviour is seen with both the chiroptic properties (Figure 2) and UV-electronic absorption. This suggested that the Cu(I)-thiolate chromophores were cleaved.

A weak electronic absorption with a maximum at 360 nm was observed which was found to originate from NO alone and without Cu-MT. In contrast

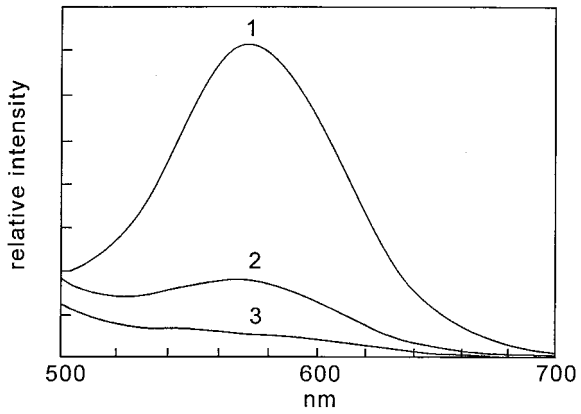

Fig. 1. Luminescence emission of yeast Cu-MT at different molar ratios of NO/Cu. (1) control, (2) 1 and (3) 2. Aqueous Cu-MT (2 ml 50 μm Cu) was gassed with NO at pH 7.0, 20 °C. The spectra were run immediately after the addition of NO.

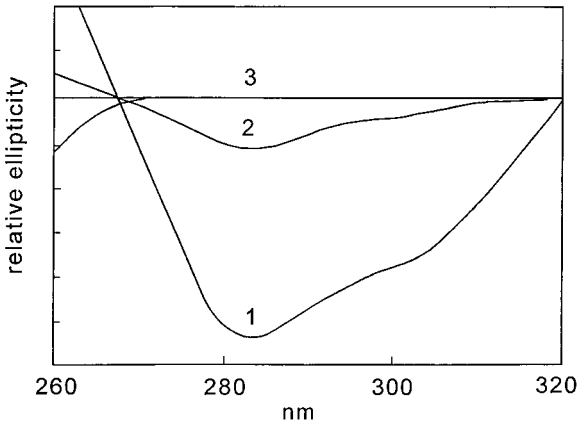

Fig. 2. Circular dichroism of yeast Cu-MT at different molar ratios of NO/Cu. (1) control, (2) 1 and (3) 2. The experimental conditions were the same as in the legend to Figure 1. The specific θ_{Cu}-value of (1) at 283 nm was -17.000 (deg $\times$ cm^2)$\times$ dmol^{-1}) and was in accordance to earlier published data (Weser & Hartmann 1988).

to Zn-MT which was treated with NO under aerobic conditions (Aravindakumar *et al.* 1999) there was no sign for S-nitrosyl formation in the present experiment which would have led to an electronic absorption in the 330 nm region. Further proof of the depletion of copper was obtained from EPR measurements which clearly show that Cu(II) is formed during the NO treatment (Figure 3). It is well known that the oxidized copper is unable to bind with the thiolate ligands of the protein.

In order to support the former conclusion of an effective copper releasing activity the NO treated Cu-MT was gelfiltrated. Indeed, separation of the reaction mixture on Sephadex G-25 revealed both the successful and complete removal of copper from the protein moiety.

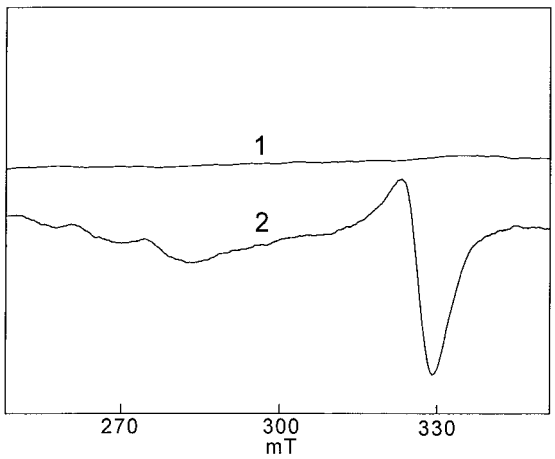

Fig. 3. EPR spectra of yeast Cu-MT. (1) untreated Cu-MT, (2) Cu-MT after gassing of the protein with a two-fold molar excess of NO expressed per equivalent of thionein-copper. Aqueous Cu-MT (0.15 mM Cu) was used in the experiment. The spectra were recorded at 100 K; modulation frequency 100 kHz; microwave power 20 mW; microwave frequency 9.24 GHz.

The question arose whether of not this copper replacement was reversible. It was intriguing to realise that some 85% of the Cu(I)-thiolate centres of the previously demetallized protein could be restored after the addition of 0.1% (v/v) 2-mercaptoethanol as monitored by both CD and luminescence emission. The progress of reconstitution proceeded slowly and was substantially finished after two hours (Figure 4). Glutathione was also effective as a reducing agent. However, in contrast to this biogenic compound, 2-mercaptoethanol was preferentially chosen to avoid overlapping of the well known perturbing spectroscopic properties of Cu(I)-glutathione. It should be pointed out that 2-mercaptoethanol in the presence of the same concentration of copper salt exhibited no Cotton effect and luminescence emission throughout the employed experimental conditions.

In addition to the above spectroscopic methods the electrophoretic behaviour of the restored Cu-MT was examined. SDS-PAGE revealed no detectable difference of the reconstituted protein compared to the untreated Cu-MT confirming that the protein moiety was not affected.

From these results it may be concluded that NO represents an efficient and reactive metabolite for the transient release of copper from Cu-MT. The reversibility of this process is both highly advantageous and economic as the protein moiety may be used re-

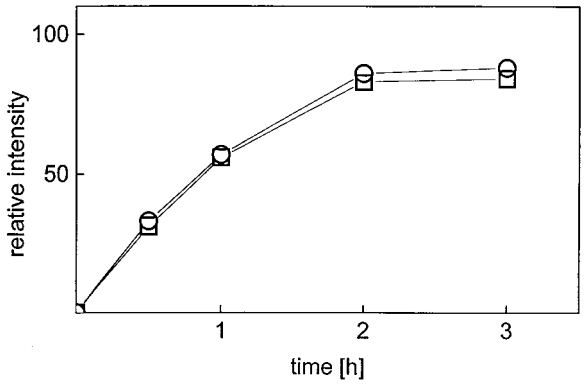

Fig. 4. Reconstitution of the Cu(I)-thiolate chromophores of NO-treated Cu-MT. Time-dependent recovery of the luminescence emission at 575 nm (◯) and dichroic amplitude at 283 nm (□) after the addition of 0.1% (v/v) 2-mercaptoethanol to the previously NO-treated protein. Relative intensities are expressed in percent of the original values.

peatedly as a copper shuttle. However, disulphides may be formed during the cleavage of the Cu-thiolate clusters. The most appropriate candidate under *in vivo* conditions for disulphide reduction certainly will be glutathione. Although the present findings are based on *in vitro* investigations they convincingly confirm earlier proposals that MT is an excellent system for sequestering copper under physiological conditions. At the same time it can be considered to be a most powerful site-directed Fenton reagent in biological defence mechanisms, e.g., the scavenging of intruding pathogenic microorganisms.

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